Protective Effects of the Active Part of Artemisia sacrorum Ledeb. against Acetaminophen-Induced Liver Injury in Mice

Hai-Dan YUAN,a,b Guang-Zhu JIN,a and Guang-Chun PIAOa,*

a College of Pharmacy, Yan-Bian University; Yanji, Jilin Province 133000, China; and b College of Pharmacy, Kyung-Hee University; Dongdaemun-ku, Hoegi-dong, Seoul 130–701, Korea.

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This study was designed to investigate the protective effects of the active part of Artemisia sacrorum Ledeb. Extract (ASE) against acetaminophen (APAP)-induced hepatotoxicity in mice. As a result, pretreated with ASE prior to the administration of APAP significantly prevented the increases of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and tumor necrosis factor-α (TNF-α) levels in serum, and glutathione (GSH) depletion, malondialdehyde (MDA) accumulation in liver tissue. In addition, ASE prevented APAP-induced apoptosis and necrosis, as indicated by a liver histopathological analysis and DNA laddering. Furthermore, according to the results from Western blot analysis, ASE markedly decreased APAP-induced caspase-3 and -8 protein expressions in mouse livers. All these results suggest that the protective effects of ASE against APAP-induced liver injury may involve mechanisms associated with its inhibitive effects of lipid peroxidation and the down-regulation of TNF-α mediated apoptosis.

Key words Artemisia sacrorum; acetaminophen; hepatotoxicity; tumor necrosis factor-α

Acetaminophen (APAP) is a commonly used antipyretic and analgesic agent.1,2 It is safe and effective when used at therapeutic doses. However, overdose of APAP can induce serious and even fatal hepatotoxicity in both experimental animals and humans.2,3 APAP is metabolized by enzymatic reaction via cytochrome P450 system and converted to a toxic metabolite known as N-acetyl-p-benzoquinone imine (NAPQI).4 NAPQI requires glutathione (GSH) for detoxification by forming its GSH-adduct.5 When having depleted the intracellular stores of GSH, excess NAPQI may react with cellular proteins and initiate the toxic response and thus lead to massive hepatocyte necrosis, liver damage or death.6

Oxidative stress is one of the proposed mechanisms and depletion of cellular GSH in the liver cells is known to play an important role in APAP toxicity.7 Malondialdehyde (MDA) is the main degradative product of lipid peroxidation processes. Oxidative stress can be evaluated indirectly by assaying the MDA level, the product of oxidative damage, which indicates the severities of membrane lipid peroxidation and cellular injury.8,9 Matsumaru et al. reported that depletion of GSH in cultured murine hepatocytes by APAP results in oxidative stress-dependent necrosis and sensitizes to tumor necrosis factor-α (TNF-α)-induced apoptosis.10 TNF-α induces apoptosis through the adaptor proteins of Fas-associated death domain (FADD) and TNFR-associated death domain (TRADD) to its receptor, which in turn leads to the activation of the initiator caspase, caspase-8. The activated caspase-8 directly activates caspase-3 which plays a pivotal role in cell apoptosis when being activated.11—14

The dried aerial part of Artemisia sacrorum Ledeb. (Compositae) is one kind of oriental folk medicine that has been traditionally used for thousands years to prevent and treat different chronic and acute hepatitis. The application of Artemisia sacrorum Ledeb. has long history and its notable hepatoprotective effects has been widely recognized in folk. However, there are no scientific studies available about protective effects of this plant medicine on liver injury. In the present study, we investigated the protective effects of the active part of Artemisia sacrorum Ledeb. on APAP-induced hepatotoxicity and elucidated the mechanisms underlying these effects in mice.

MATERIALS AND METHODS

Materials Detection kit for glutathione was purchased from Oxis International, Inc. (Foster City, CA, U.S.A), and Mouse TNF-α enzyme-linked immunosorbent assay (ELISA) kit was obtained from BD Biosciences (San Diego, CA, U.S.A). DAB (3,3′-diaminobenzidine) substrate kit for peroxidase and Vectastain® ABC kit were purchased from Vector Laboratories (Burlingame, U.S.A). Caspase-3, Caspase-8, β-actin antibodies and the peroxide-conjugated secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A). The alanine aminotransferase (ALT) and aspartate aminotransferase (AST) reagent were purchased from STANBIO Laboratory (U.S.A). D-101 macroporous resin was obtained from Tianjin Hai Guang Lian Limited Company (China). RNA extraction kits were purchased from Intron Biotechnology Inc. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.).

Preparation of Artemisia sacrorum Ledeb. Extract

The aerial part of Artemisia sacrorum Ledeb. was extracted twice with water for 2 h, 1 h respectively and the water extract (WE) was concentrated into 0.5 g (crude drug)/ml and loaded on a D-101 macroporous resin column and the column was eluted with water, 50% ethanol and 95% ethanol, respectively and thereby water eluate (WEWE), 50% ethanol eluate (50% EE), and 95% ethanol eluate (95% EE) were obtained. 50% EE was further separated into 2 parts: the precipitation (50% EEP) and the supernatant (50% EES). The yield rates of different parts were 79.0% (WEWE), 11.2% (50% EEP), 10.6% (50% EES) and 1.8% (95% EE), respectively. We have proved that 50% EES was the active part for hepatoprotective effect in our previous pharmacological experiments and therefore 50% EES was used for further research in the present study.

Animals Five weeks old male C57BL/6 mice (18—20 g)
were purchased from ORIENT BIO (Seoul, Korea). All animals were acclimatized to the laboratory environment for 1 week before the experiment. Mice were allowed to freely access to drinking water and food under constant room temperature (22 ± 2 °C) and humidity (50 ± 10%) conditions with an automatic 12 h light and 12 h dark cycle and cared for and treated in accordance with guidelines of the Committee on Care and Use of Laboratory Animal Resources, National Research Council, U.S.A. Mice were randomly divided into four groups: normal control group (Con), APAP group, *Artemisia sacrorum* Ledeb. Extract (ASE) high dose group (ASE 500 mg/kg) and ASE low dose group (ASE 250 mg/kg). The mice were fasted 11 h before intraperitoneal injection of APAP (300 mg/kg). APAP was dissolved in 20% propylene glycol. Control group mice were administered with 20% propylene glycol alone. To determine the effects of ASE, mice were pretreated orally with ASE twice per day for 5 consecutive days before the APAP injection. The final treatment of ASE was designed at 2 h before APAP treatment. For oral administration, the ASE were diluted in distilled water.

**Determination of Serum Parameters** At 12 h after APAP treatment, mice were sacrificed, and the blood samples were collected and then centrifuged at 3000 g for 15 min at 4 °C, and serum ALT, and AST levels were measured using the automated chemistry analyzer (ERBA SMARTLAB). And TNF-α level was measured by ELISA according to manufacturer’s instructions.

**Determination of Glutathione** After sacrificed, mice livers were immediately removed and instantly soaked in 4 °C, and serum ALT, and AST levels were measured using the automated chemistry analyzer (ERBA SMARTLAB). And TNF-α level was measured by ELISA according to manufacturer’s instructions.

**Determination of Malondialdehyde** Lipid peroxidation was determined by using the method reported by Buege and Aust (1978) by measuring the formation of the thiobarbituric acid-reactive substances (TBARS) spectrophotometrically.

**Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis of CYP2E1 Expression** Total mRNA was isolated from mouse livers using an Easy-Blue total RNA extraction kit according to the manufacturer’s instructions. From each sample, total RNA (10 μg) was reverse transcribed into cDNA using the Moloney murine leukemia virus transcriptase and Oligo (dT)15 primers (Promega, U.S.A.) as primers. The cDNA fragment was amplified by PCR using the following specific primers: sense strand CYP2E1, 5′-CAGGTTGCTTCTTGAGGTCCT-3′; anti-sense strand CYP2E1, 5′-CTCATGAGCTCCACACTTCT-3′; sense strand cyclophilin (CPN), 5′-ATGGTCAACCCCACTGCC-3′; anti-sense strand CPN, 5′-CTAGATGTTGTCACAAGCGG-3′. For CYP2E1, PCR was initiated a thermal cycle programmed at 95 °C for 5 min, 95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, and amplified for 35 cycle. Whereas CPN was initiated a thermal cycle programmed at 95 °C for 5 min, 95 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s, and amplified for 30 cycle. The RT-PCR products were electrophoresed on 1% agarose gels and visualized by 0.5 μg/ml ethidium bromide staining and scanning densitometry was performed with I-MAX Gel Image analysis system (Core-Bio, Seoul, Korea). CPN was amplified as a control gene.

**Histological Observation** The liver tissues were removed and fixed in 10% neutral buffered formalin and subsequently embedded in paraffin and sectioned into slices with 5 μm thickness (Leica, Wetzlar, Germany), and stained with hematoxylin & eosin for microscopic assessment (Olympus, Japan).

**Detection of Liver Caspase-3 Expression** The determinations of caspase-3 expression were performed with immunohistology staining kit (Vectastain® ABC kit, cat# PK 6101) and anti-caspase-3 antibody (Santa Cruz, sc-7148).

**Analysis of DNA Fragmentation** The liver tissues were homogenized in lysis buffer (100 mM NaCl, 10 mM Tris–HCl, 25 mM ethylenediaminetetraacetic acid (EDTA), 0.5% sodium dodecyl sulfate (SDS), pH 8.0). DNA fragmentations were separated by electrophoresis on 2% agarose gel containing ethidium bromide 0.1 mg/ml and visualized and photographed in the I-MAX Gel Image analysis system (Core-Bio, Seoul, Korea).

**Western Blot Analysis** Protein extracts were prepared in protein extraction kit (Intron Biotechnology Inc., Seoul, Korea). Lysates (30 μg) were electrobotted onto a nitrocellulose membrane following separation on a 12% SDS-polyacrylamide gel electrophoresis. Blotted membranes were incubated for 1 h with blocking solution (tris-bufffered saline/Tween 20. TBST) containing 5% skin milk (w/v) at room temperature, followed by incubation overnight at 4 °C with 1:2000 dilution of caspase-3, caspase-8, β-actin primary antibody (Santa Cruz Biotechnology, U.S.A.). Membranes were washed four times with 0.1% TBST and incubated with 1:3000 dilution of horseradish peroxidase-conjugated goat anti-rabbit or donkey anti-rabbit immunoglobulin G (IgG) secondary antibody for 1 h at room temperature. Membranes were washed four times in TBST and then developed by ECL (Amersham, Sweden).

**Statistical Analysis** Data are expressed as mean values±S.E. and comparisons of data have been done by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test for multiple comparisons. Mean values were considered significantly different when p<0.05.

**RESULTS**

**Effects of ASE on Serum ALT and AST Levels** The protective effects of ASE on the APAP-induced serum ALT and AST levels are shown in Fig. 1. The administration of APAP markedly increased serum ALT and AST levels, which reached 144.0±35.2 U/l (p<0.01) and 236.9±37.1 U/l (p<0.01), respectively, while the Con group mice had levels of 24.8±3.8 U/l and 77.2±13.5 U/l, respectively. However, pretreatment with ASE reduced both serum ALT and AST levels in a dose-dependent manner. Serum ALT levels were significantly decreased by 60.8% (p<0.01) in ASE250 group and by 75.3% (p<0.01) in ASE500 group compared to...
APAP. Serum AST levels were also decreased by 41.5% (p<0.01) in ASE250 group and by 43.7% (p<0.01) in ASE500 group compared to APAP.

Effects of ASE on Hepatic GSH and MDA Levels
GSH, one of the most important antioxidant molecules, could scavenge NAPQI which is a toxic metabolite of the APAP. Thus hepatic GSH levels were measured. 12 h after APAP administration, APAP group had significantly decreased GSH concentration by 58% (p<0.01) compared with that of Con group. However, pretreatment with ASE significantly recovered the APAP-induced GSH depletion to 126.9±5.0 (p<0.05) and 171.3±26.5 (p<0.01), respectively in ASE250 group and ASE500 group (Fig. 2A).

The concentrations of hepatic MDA were significantly increased in the APAP group (260.9±33.0 pmol/mg protein, p<0.01), when compared with the Con group (92.1±17.3 pmol/mg protein). However, pretreatment with ASE showed significant decrease in MDA levels in a dose-dependent manner (Fig. 2B).

Effects of ASE on Serum TNF-α Levels
TNF-α is an important proinflammatory cytokine, and some studies suggest its involvement in progression of APAP-induced hepatotoxicity. Therefore, we measured TNF-α concentrations in mouse sera. Administration of APAP markedly increased serum TNF-α levels (243.1±15.6 pg/ml, p<0.01), as compared to the Con group (166.4±6.5 pg/ml). Pretreatment with 250 mg/kg or 500 mg/kg of ASE reduced serum TNF-α levels by 21.4% (p<0.01) and 27.6% (p<0.01), respectively (Fig. 3).

Effect of ASE on CYP2E1 Gene Expression
To evaluate the protective effects of ASE against APAP-induced liver injury, expressions of CYP2E1 mRNA level were monitored by RT-PCR. As shown in Fig. 4, ASE pretreatment markedly decreased APAP-induced CYP2E1 gene expressions in a dose-dependent manner.

Effects of ASE on Histopathology and Immunohistochemistry
To confirm the protective effect of ASE on APAP-induced liver tissue damage, we performed histological examination. Histological observation revealed that mild to moderate multifocal degeneration and occasional periacinar necrosis were presented in APAP group liver tissues. The necrosis started from the surrounding of central veins and spread to the midzonal region. However, the sections from liver tissues of mice pretreated with ASE showed much less area of necrosis compared with the APAP group (Fig. 5A). Immunohistochemical staining for caspase-3 expression was focused in the periacinar region of the hepatocytes. Only treatment with APAP revealed strong expression of caspase-3, whereas in contrast, hardly any expressions were observed in the Con group. Pretreatment with ASE groups showed...
great decrease of caspase-3 expression, compared with the APAP group (Fig. 5B).

**Effects of ASE on Mouse Hepatocyte Apoptosis** To examine the protective effects of ASE on APAP-induced hepatocyte apoptosis, genomic DNA fragmentation was assessed in the liver tissues as shown in Fig. 6. Genomic DNA fragmentation was observed in the livers of mice 12 h after the treatment with APAP. However, very little DNA fragmentation was observed in the livers of mice pretreated with ASE.

**Effects of ASE on Caspase-3 and Caspase-8 Expressions** To further investigate the mechanism of ASE on APAP-induced liver injury, we examined caspase-3 and caspase-8 protein expressions by Western blotting analysis. As shown in Fig. 7, strong increases in the levels of caspase-3 and caspase-8 resulted from APAP administration. However, pretreatment with ASE significantly suppressed the APAP-induced caspase-3 and caspase-8 protein expressions.

**DISCUSSION**

Plants have long been used as therapeutic purposes, and many of the currently available drugs are directly or indirectly derived from plants.\(^\text{17}\) The use of traditional plant medicines has been practiced for thousands years by mankind, and despite a general insufficiency of supportive evidence concerning therapeutic efficacies, herbal medicine usage continues to increase. *Artemisia sacrorum* Ledeb. is used in China and Korea as a traditional treatment for different chronic and acute hepatitis. This is the first study to report the protective effects of *Artemisia sacrorum* Ledeb. on APAP-induced liver injury.

In the present study, we demonstrated the protective effects of active part of *Artemisia sacrorum* Ledeb. on APAP-induced hepatotoxicity and elucidated the mechanisms underlying the effect in mice. Hepatocellular degeneration and necrosis along with elevated serum ALT and AST levels were indicative of hepatotoxicity.\(^\text{18}\) In our present study, pretreatment with ASE significantly reduced APAP-induced necrotic liver damage in a dose-dependent manner and suggested that ASE may have a hepatoprotective effect, as based on its ability to reduce APAP-induced serum ALT and AST levels.

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**Fig. 5.** Microscopic View of the Liver Section Obtained from Con, APAP, ASE250 and ASE500 Treated Mice

(A) H&E staining, (B) caspase-3 immunohistochemical staining. ×200. The mice were pretreated with ASE (250, 500 mg/kg, p.o.) twice daily for 5 consecutive days. The control mice were given distilled water. 2 h after the final treatment, the mice were treated with APAP (300 mg/kg, i.p.). Mice were euthanized 24 h after the APAP-administration.

**Fig. 6.** Effects of ASE on DNA Fragmentation of the Liver in APAP-Induced Liver Injury

The mice were pretreated with ASE (250, 500 mg/kg, p.o.) twice daily for 5 consecutive days. The control mice were given distilled water. 2 h after the final treatment, the mice were treated with APAP (300 mg/kg, i.p.). DNA was extracted from liver tissues of mice 24 h after APAP administration. DNA 2 μg was loaded in the 2% agarose gel containing ethidium bromide 0.1 mg/ml and separated by electrophoresis.

**Fig. 7.** Effects of ASE on APAP-Induced Caspase-3 and Caspase-8 Protein Expressions in Mice Livers

The mice were pretreated with ASE (250, 500 mg/kg, p.o.) twice daily for 5 consecutive days. The control mice were given distilled water. 2 h after the final treatment, the mice were treated with APAP (300 mg/kg, i.p.). After 24 h intoxication, caspase-3 and caspase-8 expression in mice liver were measured by Western blot analysis.
levels (Fig. 1).

APAP is a commonly used agent to induce liver injury chemically. APAP evokes hepatotoxicity after its biotransformation to a reactive metabolite, known as N-acetyl-p-benzoquinone imine (NAPQI). APAP is metabolized by enzymatic reaction via cytochrome P450 system and converted to NAPQI.4) NAPQI, a toxic metabolite of APAP, is detoxified by GSH to form APAP-GSH adducts.5) Because GSH plays an important role in the antioxidant defense system,9 it becomes the key determinant in the APAP-induced hepatotoxicity. In the present study, 12 h after APAP administration, the contents of GSH in the APAP group mice were significantly suppressed, when compared with Con group mice. And as expected, pretreatment with ASE restored GSH levels in a dose-dependent manner (Fig. 2A). MDA level is an important indicator of lipid peroxidation. In our present study, it has been demonstrated that MDA level was markedly higher in the APAP group in comparison with the Con group. However, pretreatment with ASE significantly inhibited MDA level (Fig. 2B). Therefore, it is suggested that ASE has potential beneficial effects in oxidative stress and lipid peroxidation. Moreover, the combined results obtained from ALT, AST, MDA and GSH indicated indirectly that the mechanism of hepatoprotective effect of ASE may involve the inhibition of the P450-mediated bioactivation of APAP to NAPQI.

The toxic response of APAP is primarily initiated by NAPQI. However, evidence suggests that cytokines produced by inflammatory cells contribute to the APAP-induced hepatotoxicity. TNF-α is a multifunctional cytokine, which can reduce enzyme activities by down-regulating cytochrome P450 (CYP) expression.20) Recent studies have been reported that bioactivation of APAP to NAPQI is mediated by CYP2E1, CYP1A2, CYP3A4, and CYP2A6 in mice.21) TNF-α is a pleiotropic proinflammatory cytokine that mediates the acute-phase responses in the liver associated with an inflammatory response.7) The role of TNF-α in the mediation of APAP induced liver injury is controversial. Matsumaru and Masubuchi reported that TNF-α has an important role for expression of hepatotoxicity by APAP in vitro and in vivo.3,16) On the other hand, Boess et al. reported that APAP induced hepatotoxicity is not reduced in TNF-α null mouse.20) Moreover, Blazka et al. reported that an administration of neutralizing antibody of TNF-α and IL-1 does not suppress APAP-induced hepatotoxicity in mice.22) This differential response suggests that TNF-α and related mediators expose time were involved with TNF-α’s aggravation or alleviation of the liver toxicity induced by APAP. Numerous studies indicate that cytokines such as TNF-α, interleukin (IL)-1α and interferon (INF)-γ play detrimental roles in the pathogenesis of APAP-induced liver injury, whereas IL-6, IL-10 and IL-13 play protective role in the same liver injury.23—25) Increased TNF-α has repeatedly shown to play a pivotal role in liver injury, and serum. TNF-α level was elevated in the acute liver injury as an indirect index.22) In the present study, the serum TNF-α level was markedly decreased with ASE treatment compared with the APAP group (Fig. 3). This result also indicated that the mechanism of hepatoprotective effect of ASE may involve the inhibition of the P450-mediated bioactivation of APAP to its toxic metabolite, NAPQI in the liver. Thus, to confirm whether the protective effect of ASE on APAP-induced liver injury is correlated with the inhibition of CYP, we examined one of the CYP isform-CYP2E1 gene expressions by RT-PCR. In our results, pretreatment with ASE markedly decreased APAP-induced CYP2E1 gene expressions in a dose-dependent manner. This result demonstrated that hepatoprotective effects of ASE may be caused by its ability of inhibition of CYP2E1 activity in APAP-induced liver injury (Fig. 4).

TNF-α, one of the most important proinflammatory cytokine, induces hepatocyte apoptosis and necrosis through the activation of caspase-3 and thereby leads to liver cell DNA shift.20) Results from the histological observations of H&E staining also showed that ASE decreased hepatocyte necrosis significantly (Fig. 5A). Furthermore, APAP administration markedly increased caspase-3 expression, and on the other hand, pretreatment with ASE significantly decreased expression of caspase-3 (Fig. 5B). Moreover, consistent with these histological data, apoptosis was also markedly prevented or minimized by ASE pretreatment base on DNA laddering assays (Fig. 6). In general, caspase-3 cleaves different cellular substrates and eventually results in apoptotic cell death. Caspase-3 can be directly activated via activation of initiator caspase-8 which is the most proximal caspase and can be activated by TNF-α.11—14) To further investigate the molecular mechanisms for the caspase-3 and caspase-8 activations underlying the hepatoprotective effect of ASE, caspase-3 and caspase-8 protein expressions were examined by Western blot analysis. The data demonstrated that ASE dramatically inhibited the caspase-3 and caspase-8 protein expressions in a dose-dependent manner (Fig. 7). This result suggested that pretreatment with ASE may inhibit the APAP-induced apoptosis by down-regulating the caspase-3 and caspase-8.

In summary, our results suggest that ASE has a potent hepatoprotective effect on APAP-induced liver injury in mice. The primary mechanisms underlying the protective effects of ASE might be due to its alleviation of GSH depletion and inhibition of lipid peroxidation, and inactivation of caspase-8 and caspase-3 via inhibition of TNF-α, a pro-inflammatory mediator. These results indicate that ASE could be a valuable candidate for further development for prevention and treatment of inflammatory diseases of the liver.

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REFERENCES